EFFECT OF OAT β-GLUCAN ON IN VITRO DIGESTION CHARACTERISTICS OF SET-TYPE YOGURT

***Xiaoqing Qu*[1,2]**
[1]Department of Milk and Meat Technology, Sumy National Agrarian University
160 Herasima Kondratieva St., 40021, Sumy, Ukraine
[2]Department of Food Science, Henan Institute of Science and Technology
Hualan Rd., 453003, Xinxiang, China
https://orcid.org/ 0000-0003-3340-7336

Bo Li
Department of Food Science, Henan Institute of Science and Technology
Hualan Rd., 453003, Xinxiang, China
https://orcid.org/0000-0003-4543-0090

Wei Yang
Department of Food Science, Henan Institute of Science and Technology
Hualan Rd., 453003, Xinxiang, China
https://orcid.org/0000-0002-5230-0721

Yuliya Nazarenko*
Department of Milk and Meat Technology, Sumy National Agrarian University
160 Herasyma Kondratjeva str., 40021, Sumy, Ukraine, nazarenko.sumy@gmail.com
https://orcid.org/0000-0003-4870-4667

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Abstract
The main objective of the study was to evaluate the effect of added 0.3% (w/w) oat β-glucan (OG) in set-type yogurt on its protein digestion using an in vitro gastrointestinal model. During gastric digestion phase, the amount of soluble proteins and peptides increased to 25% and 40% for control yogurt (yogurt without OG) and 0.3% OG yogurt, respectively. Buccal digestion has little effect on the structure of yogurts, while large spherical vesicles were formed for both control yogurt and 0.3% OG yogurt after gastric digestion. The presence of 0.3% OG promoted the hydrolysis of yogurt in the gastric digestion phase and caused higher antioxidant activity. Compared with that of control yogurt, the inhibition of cholesterol solubility of 0.3% OG yogurt showed no differences after buccal digestion but significantly higher after gastrointestinal digestion (21.3% for gastric and 22.7% for intestinal digestion). Overall, this study enhances the understanding of digestion characteristics of 0.3% OG-fortified set-type yogurt and provides a theoretical basis for the development of this kind of dairy products.

Keywords
oat β-glucan; yogurt; in vitro digestion; antioxidative activity; cholesterol solubility

Introduction

Yogurt is a kind of dairy product fermented by two kinds of lactic acid bacteria (Lactobacillus bulgaricus and Streptococcus thermophilus). It has received much attention from consumers due to the high nutritional value and biological benefits. In addition, it is considered to influence some regulatory systems (such as glucose and lipid metabolism), reduce blood pressure, promote insulin secretion, and maintain the body weight, etc. [1–5].
Yet, these nutritional and biological functions of yogurt are closely related to their digestion process. The bioactive peptides, existing in the amino acid sequence of protein, can be released and activated only through enzymatic hydrolysis during the digestion process. Especially, some branched-chain amino acids, which can influence several postprandial metabolic responses, are present in digested dairy products [6].
Oat β-glucan (OG) is an important soluble dietary fiber, consisting of linear chains of β-D-glucopyranosyl units linked with (1→3) and (1→4) linkages [7]. It has many biological activities, such as enhancing antioxidant activity,

reducing blood lipid, preventing cardiovascular diseases, regulating gastrointestinal environment and cholesterol level in body [8–11]. OG is also well-known for its thickening, stabilizing, emulsifying and gelling properties to maintain the stability of ingredients [12]. More importantly, it has been found that, OG has prebiotic properties and could selectively enhance activity and raise growth of probiotic bacteria (such as lactobacilli and bifidobacteria). So, OG can be used as a texturizer, fat replacer, and prebiotic in enhancing the physical characteristics and nutraceutical qualities of yogurt [13–15]. According to our previous study, the addition of 0.3% (w/w) OG could maximize the quality characteristics of set-type yogurt, and shorten the fermentation time [16]. Due to the addition of OG, the digestion characteristics (e.g., the degree of hydrolysis) of set-type yogurt and the structural and functional properties of proteins or peptides after digestion (e.g., molecular weight, charge and hydrophobicity, etc.) may be changed. However, there are relatively few studies on the effect of 0.3% OG on the *in vitro* digestion characteristics of set-type yogurt, which will limit the application of this type of yogurt.

In vitro digestion models have been designed to study the structural changes, digestibility/degradation, and digestion characteristics of food components under simulated gastrointestinal conditions [17]. Through these models, the digestion characteristics of food systems, such as plant-, dairy-, and emulsion-based foods, has been successfully studied.

So, the main objective of this study was to investigate the effect of 0.3% OG on the in vitro digestion characteristics of set-type yogurt by an in vitro gastrointestinal (GI) model. The proportion of yogurt soluble proteins and peptides after digestion was measured. The microstructural morphology and particle size of yoghurts after digestion were characterized by optical microscopy and dynamic light scattering, respectively. The antioxidant activities and inhibition of cholesterol solubilization into micelles were also evaluated.

Methods

Pure milk was purchased from Yili Industrial Group Co. Ltd (Neimenggu, China). Oat β-glucan (95% purity) were purchased from Zhongkang Food Co., (Guangzhou, China). Starters: Streptococcus thermophilus and Lactobacillus bulgaricus (Lactobacillus dechellii Bulgarian subspecies) (viable bacteria count was about 1×10^9 CFU/g) were purchased from Danisco (China) Co., Ltd, (Shanghai, China). Amylase (1000–3000 U/mg protein), pepsin from porcine stomach mucosa (1:60,000), pancreatin from porcine pancreas (8 × USP) and sodium deoxycholate, cholesterol, oleic acid, phosphatidylcholine, and bile from bovine were obtained from Sigma Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade.

Yogurt preparation

Yogurt fortified with OG (0.3%, w/w) was prepared according to our previously reported method [16]. 0.3% of OG was added to pure milk. After stirring, the milk was sterilized at 95 °C for 5 min, and then cooled to 43 °C, added the starters (containing Streptococcus thermophilus and Lactobacillus denderi Bulgarian subspecies), fermented at 43 °C for 5 h, and stored at 4 °C for 24 h.

In vitro digestion

The simulated gastrointestinal digestion study was performed according to the methods described by Minekus et al. and Asensio-Grau et al. with some minor modifications [18,19]. The gastrointestinal digestion process was conducted as follows:

a. Buccal stage: Simulated salivary fluid at pH 7.0 was added to yogurts in a ratio 1:1 (w/v) under gentle stirring using a kitchen blender for 2 min at 37 °C. Human α–amylase was added as a part of the salivary fluid to reach a desired concentration (75 U/mL) in the saliva mixture.
b. Gastric stage: After the buccal stage, simulated gastric fluid (pH 3.0) was added to tubes in a 1:1 (v/v) ratio including pepsin, reaching a desired concentration (2000 U/ml) in the gastric mixture. The pH of yogurts was adjusted to 2.0–2.5 with 2.5 M HCl. Then, the sample solutions were mixed thoroughly and incubated at 37 °C for 30 min by a shaking incubator. After the incubation, the sample solutions were centrifuged at 10,000 g for 10 min (Centrifuge 5430R, Hamburg, Germany). Further analyses were conducted for the collected supernatant.
c. Duodenal stage: After the gastric stage, simulated intestinal fluid containing 2 mL of porcine pancreatin and 1 mL of bile acid mixture (pH 6.0 or 7.0) was added in 1:1 (v/v) ratio to tubes containing the gastric chime. The pH of sample solutions was adjusted to 7.0 with 4 M NaOH. The sample solutions were incubated at 37 °C for 90 min by a shaking incubator. After the incubation, the sample solutions were centrifuged at 10,000 g for 10 min (Centrifuge 5430R, Hamburg, Germany). Further analyses were conducted for the collected supernatant.

Total soluble protein content of digested samples

Protein contents in control yogurt (yogurt without OG) and in supernatants from digested yogurts (after centrifugation) were determined with the bicinchoninic acid protein assay kit (Pierce Company). The content of soluble protein in digested yogurts was expressed as percentage (%) of total protein in undigested ones. All experiments were performed in triplicate.

Optical microscopy

The microstructure of control yogurt and digested yogurts was observed by optical microscopy (Axio Vert.A1, Carl Zeiss), according to the previous works [20]. Yogurt samples were put between glass slides and immediately observed at a magnification of 100× at room temperature. All experiments were performed in triplicate.

Particle size and size distribution

The particle sizes of control yogurt and digested yogurts were measured by dynamic light scattering using a Zetasizer Nano ZS90 (Malvern Instruments Ltd, Worcestershire, U.K.). Particle size was obtained by the Stokes-Einstein equation. The polydispersity index (PDI), representing the distribution of particle size, was also reported. Before measurement, all samples were diluted by 1:5 (v/v) with deionized water at the corresponding pH values and then equilibrated for 2 min inside the instrument at 25 °C. Data were collected over at least 20 sequential readings. All experiments were performed in triplicate.

Antioxidant activities

The 2, 2-diphenyl-1-picryhydrazyl (DPPH) assay for antioxidant activities of control yogurt and digested yogurts were determined according to the method of Unal et al. [21] with minor modifications. Briefly, 2 mL of each yogurt sample and 2 mL of 0.1 mM DPPH solution (90% methanol) were mixed and vortexed vigorously. Then, the mixtures were allowed to keep in the dark for 30 min at room temperature. Finally, solution absorbance at 517 nm was measured by an ELX800 Microplate Reader (Bio-Tek, Bedfordshire, UK). Blank samples were prepared by replacing the yogurt samples with methanol. All experiments were performed in triplicate.

The scavenging activity was determined as follows:

(1) $$\text{Scavenging Activity } (\%) = 100 \times \frac{\mathrm{A_{DPPH}} - \mathrm{A_S}}{\mathrm{A_{DPPH}}}$$

where A_S is the absorbance of the yogurt samples, and A_{DPPH} is the absorbance of the blank samples.

The 2, 2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assay for antioxidant activities of control yogurt and digested yogurts was determined according to the method proposed by Liang et al. [22]with minor modifications. ABTS radical cation was produced by reacting 7 mM ABTS with 2.45 mM potassium persulfate, stored in the dark for 15 h at room temperature. Before usage, the ABTS solution was diluted with phosphate buffered solution (pH 7.4) to get an absorbance value of 0.70 ± 0.02 at 734 nm. 0.2 mL of each yogurt sample was mixed with 3.8 mL of the prediluted ABTS solution, standing for 6 min at room temperature before measurement. All experiments were performed in triplicate.

The scavenging activity was determined as follows:

(2) $$\text{Scavenging Activity } (\%) = \left(1 - \frac{\mathrm{A_{yogurt}}}{0.700}\right) \times 100\%$$

In vitro cholesterol micelle

Cholesterol micelles were prepared following the two methods described by Kirana et al. and Ashraf et al. with some minor modifications [23,24]. An emulsion at pH 7.4, mainly containing 0.5 mM cholesterol, 10 mM sodium taurocholate, 1 mM oleic acid, 1 mM cholesterol, 132 mM NaCl, and 15 mM sodium phosphate buffer, was prepared. And then, the emulsion was treated with ultrasonic energy (400 W, 20 kHz, 20 min), and incubated at 37 °C overnight. Each yogurt sample was mixed with the emulsion and the obtained mixtures were incubated at 37 °C for 24 h. Afterwards, the mixtures were centrifuged at 8000 g for 30 min and the supernatants were

collected. Cholesterol contents in the supernatants were determined by a total cholesterol kit. All experiments were performed in triplicate.
Micelle cholesterol uptake inhibition was calculated according to the formula used by Marques et al. [25]:

$$\text{Inhibition Capacity (\%)} = (1 - C_1/C_0) \times 100\% \quad (3)$$

where C_0 is the cholesterol concentration in the micelle, and C_1 is the cholesterol concentration with peptides.

Statistical analysis
OriginPro 8.6.0 (Originlab, Northampton, MA, USA) was used for the construction of the graphs. Data were presented as means ± standard deviations of three independent experiments and analysed for significant difference by one-way analysis of variance (ANOVA) using the SPSS software, version 18.0 program (SPSS Inc., Chicago, USA).

Results and discussion
In vitro protein digestibility
Figure 1 shows the proportion of yogurt soluble proteins and peptides after the buccal, gastric and duodenal digestion phases. Overall, the amount of soluble proteins and peptides increased during digestion.

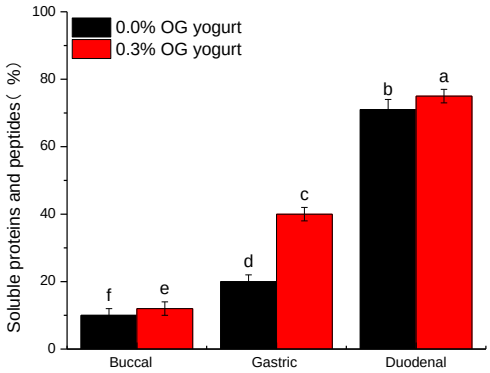

Figure 1. Soluble proteins and peptides (%) after the buccal, gastric and duodenal digestion phases. Different characters on the top of columns indicate statistically significant differences at $p < 0.05$ between different samples ($n = 3$).

The amount of soluble proteins and peptides increased slightly during buccal digestion phase. But, after the simulated gastric digestion, there was a significant ($P \leq 0.05$) increase (25% for control yogurt, 40% for 0.3% OG yogurt) of the soluble proteins. This result is in agreement with the study by Rinaldi et al. [6], who reported that due to the presence of OG, yogurts exhibited faster proteolysis, thus leading to the lower release behavior of large peptides while higher percentage of free amino acids. After the simulated duodenal digestion, the soluble proteins and peptides were slightly higher for 0.3% OG yogurt than for the control yogurt. Thus, OG addition does influence the in vitro protein bioaccessibility in yogurt, especially after the gastric step. It was reported that some polysaccharides, such as gum arabic, low-methylated pectin, and xylan, could inhibit β-lactoglobulin digestibility, due to the formation of protein-polysaccharide complexes [26]. The difference might be attributed to the different physiochemical characteristics of polysaccharides. This also may suggest that OG is more suitable for use as a functional food ingredient in enhancing the nutraceutical quality of yogurt compared to other polysaccharides.

Microstructure and particle size
To gain more structural insights, the microstructural morphologies of particles for control yogurt and 0.3% OG yogurt after buccal, gastric and duodenal digestion were observed by optical microscopy (Figure 2).
Buccal digestion has little effect on the structure of yogurts. After buccal digestion, the microstructure of control yogurt showed a clear three-dimensional protein network structure (Figure 2 (a1)). In general, during fermentation, casein aggregates form a three-dimensional network in yogurt [27]. The microstructure of 0.3%

OG yogurt also showed a denser three-dimensional network structure (Figure 2 (b1)). This could be due to the network structure formed by OG or the complexes dominated by OG-casein interactions, in good agreement with in our previous work (studied by scanning electron microscopy) [16].

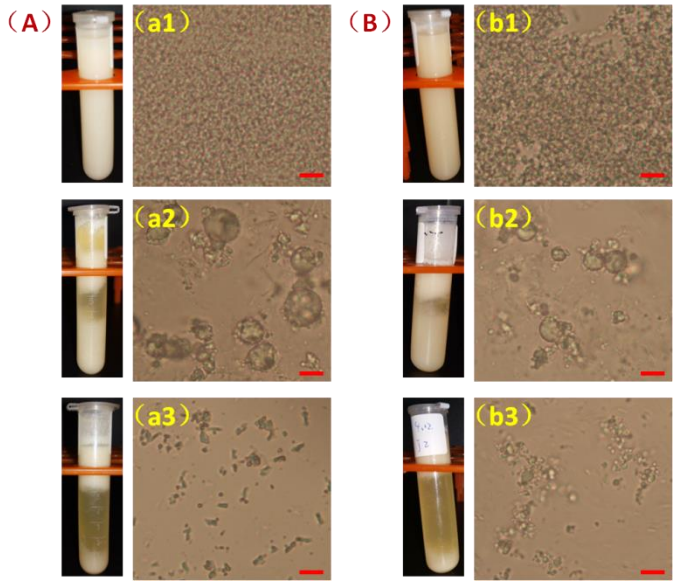

Figure 2. The microstructure of yoghurts after digestion. (A) control yogurt; (B) 0.3% OG yogurt. (a1, b1) after buccal digestion; (a2, b2) after gastric digestion; (a3, b3) after duodenal digestion.

After gastric digestion, large spherical vesicles were formed for both control yogurt and 0.3% OG yogurt (Figure 2 (a2, b2)). In general, the main role of pepsin is to enzymatically hydrolyze proteins into large peptides. These spherical vesicles should be protein aggregation caused by gastrointestinal digestion. Interestingly, the particle sizes of spherical vesicles for 0.3% OG yogurt were smaller than those for control yogurt. As far as we know, the smaller particle sizes of spherical vesicles were observed for the first time by optical microscopy. This clearly suggested that the presence of 0.3% OG caused a fast enzymatic hydrolysis, leading to a significant ($P \leq 0.05$) increase in the proportion of low-molecular-mass peptides. An earlier investigation even pointed out that after gastric digestion, intact dairy proteins remained in the control yogurt whereas less in yogurts enriched in pectin/OG, as measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis [6].
After duodenal digestion, spherical vesicles disappeared, and some small flake structure occurred instead for control yogurt (Figure 2 (a3)). This indicates complete digestion of control yogurt. In fact, Arora et al. pointed that some compounds, such as short chain fatty acids, are easier to hydrolyze by lipases under the role of bile salts [28]. In comparison, there were still some spherical particles with small particle sizes in 0.3% OG yogurt, and they are related to each other (Figure 2 (b3)). Clearly, these connected spherical particles were related to the presence of OG, as OG can interact with proteins and peptides and resist to hydrolysis by lipases to some extent [29]. This may play an important role in stabilizing and reinforcing the functional properties of these peptides. In fact, OG has been used in the food, cosmetic and pharmaceutical fields to deliver bioactive compounds [30].

It can be seen from Table 1, for control yogurt, the average particle size was as high as 7.2 μm with PDI of 0.73 after buccal digestion. This indicated that particles were aggregated with each other. After the gastric digestion, its particle size was decreased to 5.3 μm, and PDI also decreased to around 0.63. After the intestinal digestion, its particle size was about 1.2 μm, with a low PDI of 0.31. This directly indicated that yogurt was completely digested.
Differently, the particle size after buccal digestion was higher for 0.3% OG yogurt than for the control yogurt. This was obviously related to the addition of OG. But, after the gastric digestion, the particle size was decreased to 3.4 μm, and PDI also decreased to around 0.53. This again indicated that 0.3% OG addition caused an increase in the proportion of peptides during digestion, as observed *via* optical microscopy. The fast protein digestion for yogurts with OG could suggest a phase separation phenomenon between OG and protein. We hypothesize that in gastric solution, digestion conditions favor the phase separation, forming a “micro-reactor” among OG, yogurt

proteins, and enzymes. Similar result was also obtained by Rinaldi [6]. In the micro-reactor, enzymes and yogurt proteins are in close contact, thus facilitating the hydrolysis, leading to small particle sizes.
After the intestinal digestion, the particle size was slightly higher (about 1.5 μm) than that of control yogurt, with a higher PDI of 0.39. This may be due to the undigested OG.

Table 1. Particle size and PDI of yoghurts after the buccal, gastric and duodenal digestion phases. Different characters on the top of columns indicate statistically significant differences at $p < 0.05$ between different samples (n = 3).

Digestion stages	**Samples**	**Average particle size (d/nm)**	**Polydispersity (PDI)**
Buccal digestion	control yogurt	7211 ± 45^{b}	0.73 ± 0.09^{b}
	0.3% OG yogurt	8327 ± 52^{a}	0.82±0.11a
Gastric digestion	control yogurt	5319 ± 122^{c}	0.63 ± 0.08^{c}
	0.3% OG yogurt	3427 ± 53^{d}	0.53 ± 0.04^{d}
Intestinal digestion	control yogurt	1253 ± 22^{f}	0.31 ± 0.02^{f}
	0.3% OG yogurt	1503 ± 42^{e}	0.39 ± 0.05^{e}

Antioxidant activities

Yogurt is an important source of food derived protein. In the digestion process, yogurt can release some functional active substances from milk protein, especially some bioactive peptides with good antioxidant properties. At present, DPPH assay and ABTS assay are often used to evaluate the antioxidant activity of functional foods. Here, the two methods were used to evaluate the antioxidant activity of set-type yogurt throughout digestion.
For the DPPH assay, as shown in Figure 3 (a), after buccal digestion, both control yogurt and 0.3% OG yogurt showed certain DPPH radical scavenging activity. Moreover, 0.3% OG yogurt had stronger antioxidant capacity, which can be attributed to the antioxidant activity of OG. OG has been reported to significantly inhibited the fat oxidation of low-fat beef patties [31].
After the gastric digestion, it was increased by 25% compared with that after buccal digestion, indicating that some active components were produced during gastric digestion. This result was similar to other report [10]. Interestingly, 0.3% OG yogurt exhibited higher DPPH scavenging ability (43%) than the control. The result clearly indicated that the presence of OG promoted the yogurt protein to produce more antioxidant components, giving enhanced antioxidant properties.
After the intestinal digestion, the DPPH radical scavenging activity of yogurts cannot be detected. In the study, DPPH was dissolved in methanol, which is suitable for the determination of hydrophilic compounds, not suitable for lipophilic compounds [32,33]. It was speculated that the lipophilic compound after the intestinal digestion may interfere with the determination. Detailed reasons need further study.
The results of ABTS assay were similar to DPPH assay for buccal and gastric digestion (Figure 3 (b)). In comparison, the ABTS radical scavenging capacity of yogurts can be detected after the intestinal digestion. It was further improved (43% for control yogurt and 59% for 0.3% OG yogurt). Clearly, the antioxidant activity of yogurt was further improved.

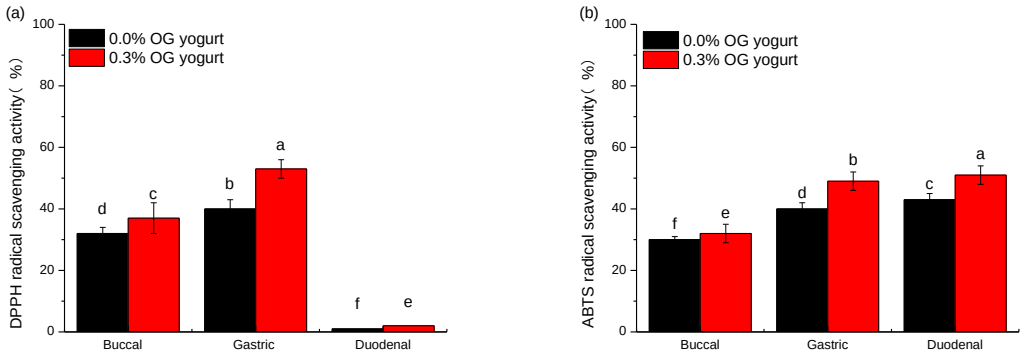

Figure 3. DPPH (a) and ABTS (b) radical scavenging capacity of yogurts after the buccal, gastric and duodenal digestion phases. Different characters on the top of columns indicate statistically significant differences at $p < 0.05$ between different samples (n = 3).

In short, through DPPH and ABTS assays, the antioxidant activity of yogurt was mainly produced during the gastric digestion phase, and 0.3% OG could further improve the antioxidant activity of yogurt by promoting the beneficial enzymatic hydrolysis. And, compared with DPPH assay, ABTS assay is more suitable for evaluating the antioxidant activity of set-type yogurt during digestion.

In vitro cholesterol micelles

In most developed countries and a few developing countries, cardiovascular diseases are considered to be the first leading cause of death and morbidity, and a major contributor to greatly reduced quality of life [34,35]. Prevalent cases of total cardiovascular diseases nearly doubled from 271 million in 1990 to 523 million in 2019, and the number of cardiovascular diseases deaths steadily increased from 12.1 million in 2019 [35]. The risk of cardiovascular diseases can be reduced 2%-3% by every 1% decrease of serum total cholesterol. Dietary cholesterol need to be digested by various enzymes under salivary and gastrointestinal conditions to form micellar solution with triglycerides, phospholipids and bile acids before it can be transported into intestinal mucosal cells [36]. So, the cholesterol lowering effect was evaluated by an in vitro cholesterol micelle model [37].

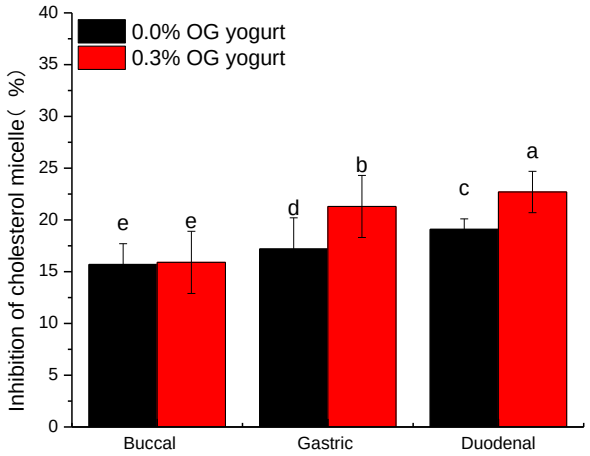

Figure 4. Percent inhibition of the micellar cholesterol solubilization of yogurts after the buccal, gastric and duodenal digestion phases. Different characters on the top of columns indicate statistically significant differences at $p < 0.05$ between different samples (n = 3).

For control yogurt, the inhibition of cholesterol solubilization into micelles gradual increased throughout digestion (15.7% for buccal digestion, 17.2% for gastric digestion, and 19.1% for intestinal digestion) (Figure 4). Clearly, this could be related to the released bioaccessible peptides and amino acids.

Interestingly, compared with that of control yogurt, the inhibition of cholesterol solubility of 0.3% OG yogurt showed no differences after buccal digestion but significantly ($P \leq 0.05$) higher after gastrointestinal digestion (21.3% for gastric digestion and 22.7% for intestinal digestion). This can be related to the presence of OG. On the one hand, OG could influence the type and conformation of amino acids present in peptides, facilitating the production of more hydrophobic amino acids. It has been reported that peptides with more hydrophobic residues can compete with cholesterol molecules through rearrangements [37]. On the other hand, OG could compete with cholesterol to enter the micelle solution and reduce the cholesterol solubility.

Yet, for OG-fortified yogurt, its property of inhibition of cholesterol solubilization into micelles may not be solely due to the two reasons above. Recent studies reveal that the gut microbiota plays a significant role in lowering cholesterol in humans [38–40]. Importantly, OG has the ability to modulate the gut microbiota in human [41,42]. So, further studies are necessary to evaluate the effect of specific interactions between digested yoghurt components and the human gut microbiota on the inhibition of cholesterol solubilization into micelles.

Impact

Worldwide, the number of chronic diseases such as hypertension, diabetes, dyslipidemia and overweight/obesity caused by unhealthy lifestyles is increasing. How to improve the health of this kind of people is the research direction of food scientists in recent years. Regular consumption of yogurt is one of the most advantageous strategies to solve these problems. This is mainly because of the functional proteins and peptides from digested yogurt. Interestingly, after gastrointestinal digestion, 0.3% OG-fortified set-type yogurt exhibited better functional properties in comparison to control yogurt. Thus, the 0.3% OG-fortified set-type yogurt can be developed as a new functional fermented dairy product to respond to consumer demand for healthier and more sustainable products.

Conclusions

In the study, the protein digestion of 0.3% OG-fortified set-type yogurt was evaluated using an in vitro gastrointestinal model. In comparison with control yogurt, the amount of soluble proteins and peptides increased throughout digestion for 0.3% OG yogurt. The presence of 0.3% OG promoted the hydrolysis of yogurt in the gastric digestion phase and caused higher antioxidant activity and higher inhibition of cholesterol solubility. Overall, this study provides a theoretical basis for the development of the 0.3% OG-fortified set-type yogurt.

Conflict of interest

There are no conflicts to declare.

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